Rhinology, 36, 101–103, 1998

Immunohistochemical localisation of tear lipocalin in human nasal mucosa*

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SUMMARY

Lipocalins are low molecular weight soluble proteins, a sub-class of this family are the odorant binding proteins (OBPs) which are postulated as having an important role in the perireceptor events of olfactory transduction, though their specific physiological function has yet to be defined. From the nasal mucus of normal subjects we recently isolated a 19kDa protein, the amino-acid sequence of which, limited to the first 20 residues, is identical to that of tear lipocalin. In this study we performed an immuno-histochemical investigation on the nasal localisation of this protein, using tissue specimens taken from the inferior (27 samples), middle (7 samples) and superior turbinates (6 samples) in 27 patients undergoing septoplastic surgery. The protein was detected in the sub-epithelial tubulo-acinar glands of the nasal mucosa, particularly in the mucoserous glands, in 74% of the specimens taken from the inferior turbinate, in 71.4% of those from the middle turbinate and in 66.6% of the samples of superior turbinate tissue. The homogeneous distribution of this protein in the nasal mucus could imply that it functions as a general protection agent rather than as an odour carrier or transducer.

Key words: lipocalins, odorant-binding proteins, human nasal mucosa, immunohistochemistry

INTRODUCTION

Lipocalins are low molecular weight soluble proteins, carriers for hydrophobic ligands in aqueous biological fluids (Sansom et al., 1994; Flower, 1996). A sub-class of this family is represented by odorant-binding proteins (OBPs), so called for their affinity towards small volatile molecules. The OBPs in vertebrates are acidic proteins of about 20 kDa, secreted in high concentration in the nasal mucus (Pelosi et al., 1982, Pelosi, 1994, Pelosi, 1996). These proteins have been purified from several species and some of them have been characterised both in terms of ligand-binding specificity and amino-acid sequence. The three-dimensional structure of bovine OBP has been defined and shows the typical β-barrel motif common to other lipocalins (Bianchet et al., 1996, Tegoni et al., 1996).

It is assumed that these proteins play an important role in the perireceptor events of olfactory transduction, but their specific physiological function has not been clearly defined (Pelosi, 1994, 1996). Several models and hypotheses have been suggested, indicating these proteins as carriers of odorants towards or away from the membrane-bound olfactory receptors (Pevsner and Snyder, 1990), as buffering and filtering agents or as active transducers of the olfactory messages (Pelosi, 1994). According to this last hypothesis, OBPs activate the olfactory receptors only when in complex with the odorant molecules, with a mechanism similar to that used by bacteria to detect the presence of sugars (Koshland, 1981; Stewart and Dahlquist, 1987).

While OBPs have been identified in very high concentrations in the nasal mucosa of several mammal species, the human mucus failed to reveal the presence of proteins with similar odour binding characteristics. Instead, we have recently isolated a 19 kDa protein (Scalfari et al., 1997), which, based on its amino terminal sequence and chemical characteristics, appears to be identical or extremely similar to another lipocalin, present both in lachrymal glands (tear lipocalin, Redl et al., 1992; Garibotti et al., 1995) and in von Ebner's glands (VEG protein, Schmale et al., 1990; Blaker et al., 1993; Garibotti et al., 1995) of the circumvallate papillae of the tongue. Recently, the same protein has also been identified in the prostate (Holzfeind et al., 1996).

This protein was detected by our group in the nasal mucus of healthy subjects, while searching for OBP-like proteins (Scalfari et al., 1997). It was purified to homogeneity by a combination of anion exchange chromatography and gel filtration and appeared to be present as a monomer in its native state. It exhibits an apparent molecular weight in SDS-PAGE of 19 kDa and an aci-

* Received for publication April 2, 1998; accepted May 28, 1998

dic behaviour. The amino acid sequence, limited to the first 20 residues, is identical to that of tear lipocalin. By means of immunohistochemistry, we also showed that this protein is synthesised in the nasal mucosa. In this paper we further investigate the sites of production of tear lipocalin in the nasal area of human healthy subjects.

MATERIAL AND METHODS

Samples of nasal mucosa

For the immunohistochemical investigation in this study we used tissue specimens obtained during septoplastic surgery performed on 27 patients (15 males and 12 females) whose ages ranged from 18 to 49 years (mean 28.5 ± 12.7 SD), the specimens were taken from the inferior nasal turbinates in all 27 cases, as well as from the middle turbinates in 7 and from the superior ones in 6. All these patients were affected with congenital or post-traumatic septal deviation. Smokers or patients whose profession exposed them to dust or irritating vapours were not included in this study. We also excluded patients who used topical nasal drugs. The cases were selected from those referred to the ENT Clinic of Pisa University, on the basis of the quality of the tissue and by taking into account the representativeness of the sample.

Antiserum

The anti-serum was prepared by immunising a rabbit with the 19 kDa protein, purified from the tongue of the *Cercopithecus aethiops* monkey, following standard protocols, as previously described (Scalfari et al., 1997). The crude anti-serum was fractionated by precipitation with ammonium sulphate at 45% saturation. The pellet was dissolved in PBS and used for Western blot and immunohistochemical experiments.

Immunohistochemistry

For immunohistochemical staining, blocks of formalin fixed, paraffin embedded tissues were sectioned into 5 μm thick slices, collected on gelatin-coated slides and dried overnight at 37°C. All subsequent procedures were performed at room temperature. After deparaffinisation and rehydration in graded alcohols, the sections were incubated in methanol containing 0.3% H_2O_2 for 15 minutes to inhibit endogenous peroxidase. The slides were then washed in 0.1 M phosphate buffer saline (PBS) at pH 7.2 for 10 minutes and incubated in 10% normal goat serum for 15 minutes, to block any specific reaction of the second antibody. All the serum and antibody solutions were in PBS containing 0.1% bovine serum albumin (BSA). Thereafter, the sections were incubated for 30 minutes with the primary antibody. Reaction was carried out with an avidin-biotin kit (Vectastain Elite, Vector, Burlingame, CA) according to the manufacturer's instructions. A positive reaction was a brown colouring visualised with 0.06% diaminobenzene (Poloscience) and 0.01% H_2O_2. After a brief counter-staining of the cell nuclei with haematoxylin, the sections were dehydrated in ethanol, cleared in xylene and then mounted. Negative controls consisted in the omission of the primary antibody. The preparations which did not show positive reaction were subsequently re-examined individually with serial sections of 5μ: every third slide was analysed for a total of ten sections for each sample. Negative and positive controls were included in every experiment. The histochemical analysis was performed microscopically by two separate investigators.

RESULTS

A first immunohistochemical investigation was performed with the antiserum against the VEG protein, purified from the tongue of the primate *Cercopithecus aethiops*. Out of 40 samples examined from the same number of subjects, 21 gave positive reaction (16 in the inferior turbinate, 3 in the middle and two in the superior). The remaining 19 cases, negative at a first screening, were distributed as follows: 11 from the inferior turbinate, 4 from the middle and 4 from the superior. Seventeen of these, when re-examined through series of sections, gave the following results: inferior turbinate: 4 positive out of 9; middle turbinate: 2 positive out of 4; superior turbinate: 2 positive out of 4. Hence, positive results were obtained in 29 subjects out of 40, as reported in Table l.

Table 1. Immunohistochemical reactivity of the policlonal antibody in nasal turbinates (superior, middle and inferior, respectively).

Samples	Total	Positive	Negative
Superior turbinate	6	4 (66.6%)	2 (33.3%)
Middle turbinate	7	5 (71.4%)	2 (28.5%)
Inferior turbinate	27	20 (74%)	7 (26%)

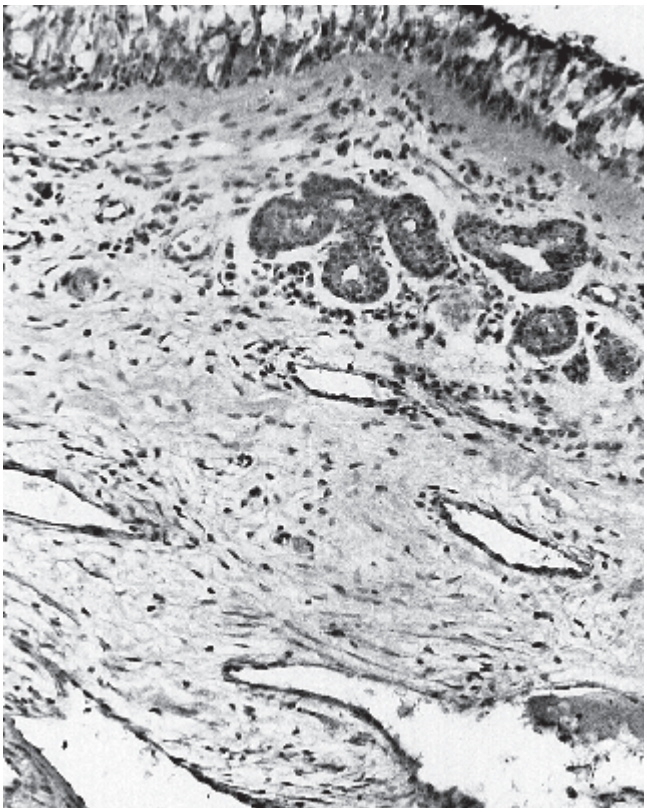

Figure 1. Intense, diffuse cytoplasmatic immunoreactivity in the mucoserous glands underlying the respiratory epithelium of the inferior turbinate; 100×.

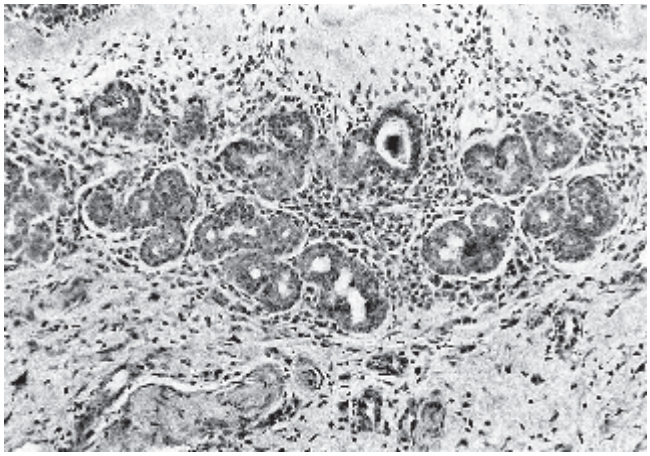

Figure 2. Cytoplasmatic positivity in numerous mucoserous glands in the middle turbinate; 200×.

We observed positive staining of the sub-epithelial tubulo-acinar glands of the nasal mucosa, in particular, the mucoserous glands appeared intensely stained in the cytoplasm of adenomere cells and in the luminal secretions. In only a few samples there was positive staining of the membranes while the mucus glands showed no positive reaction (Figure 1). Analysis of an enlargement of 200× confirmed the presence of a cytoplasmatic immunoreactivity in the epithelium of the mucoserous glands (Figure 2).

DISCUSSION

The immunohistochemistry experiments reported here clearly indicate that a lipocalin related to OBPs is synthesised in the nasal cavity. However, neither the tissue localisation nor the amino-acid sequence similarity with odorant-binding proteins provide evidence that this protein has a role in olfactory transduction. Ligand binding experiments, performed with several odorant molecules, failed to give positive responses. Moreover, the same protein is reported to be present in other organs and tissues, such as the lachrymal glands (Redl et al., 1992; Garibotti et al., 1995), the von Ebner's glands (Schmale et al., 1990; Blaker et al., 1993), and the prostate (Holzfeind et al., 1996). Recently, the capacity for binding medium length linear fatty acids has been measured in tear lipocalin (Glasgow et al., 1995). Moreover, some activity of thiol protease inhibition has been reported (Van 't Hof et al., 1997). These observations and the rather ubiquitous presence of this protein in mucosal tissues suggest a protective and antibacterial function.

Our immunohistochemical evaluations revealed that this protein is produced in the tubular acinar glands of the nasal conchae and particularly in the mucoserous glands, the same glands which synthesise odorant-binding proteins in other animal species (Pevsner et al., 1986; Avanzini et al., 1987). In particular, the strongest immunoreactivity was present in the tubular acinar glands underlying the respiratory epithelium, for a more readily available supply of the protein.

The homogeneous distribution of the protein in the nasal fossae, even in areas which are far removed from the olfactory region, may support the theory that the function of this protein is that of a general protection agent, rather than that of an odour carrier or transducer.

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